REPORTS

Bax and Adenine Nucleotide Translocator Cooperate in the Mitochondrial Control of Apoptosis

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The proapoptotic Bax protein induces cell death by acting on mitochondria. Bax binds to the permeability transition pore complex (PTPC), a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability. Immunodepletion of Bax from PTPC or purification of PTPC from Bax-deficient mice yielded a PTPC that could not permeabilize membranes in response to atractyloside, a proapoptotic ligand of the adenine nucleotide translocator (ANT). Bax and ANT coimmunoprecipitated and interacted in the yeast two-hybrid system. Ectopic expression of Bax induced cell death in wild-type but not in ANT-deficient yeast. Recombinant Bax and purified ANT, but neither of them alone, efficiently formed atractyloside-responsive channels in artificial membranes. Hence, the proapoptotic molecule Bax and the constitutive mitochondrial protein ANT cooperate within the PTPC to increase mitochondrial membrane permeability and to trigger cell death.

Pro- and antiapoptotic members of the Bcl-2 family have pleiotropic effects on caspase activation cascades regulated by the apoptosome, on cellular redox potentials, and on the barrier function of mitochondrial membranes (*1–9*). The proapoptotic protein Bax redistributes to and acts on mitochondria to induce cell death (*6–9*). Bax induces all mitochondrial hallmarks of apoptosis when overexpressed in cells, including the dissipation of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) (*1*) and the release of cytochrome c through the outer mitochondrial membrane (*6–9*).

These effects of Bax are the same as those of agents that open the permeability transition (PT) pore, a structure involved in the control of apoptosis (*2*). Accordingly, recombinant Bax (*10*) and atractyloside (Atr)—a PT pore–

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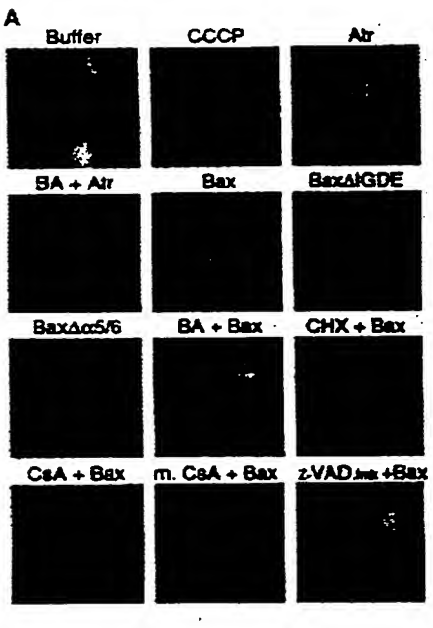
opening agent that acts on one of the PTPC components, ANT (also called ATP/ADP carrier)—induced $\Delta\Psi_m$ dissipation and nuclear apoptosis when microinjected into cells with an efficiency of ≥80% (Fig. 1A). We found no such effects for a mutant inactive Bax protein (ΔIGDE) (*10*), which lacks a motif within the BH3 domain essential for homodimerization, nor for a Bax protein (Δα5/6) lacking the putative pore-forming domain α5/6 between BH1 and BH2 (*10*) (Fig. 1A).

The mitochondrial and nuclear effects of Bax are prevented (<10%) by three prototypic inhibitors of the PT pore: cyclosporin A (CsA) (*8, 11, 12*), the nonimmunosuppressive CsA derivative *N*-methyl-4-Val-CsA (m.CsA, which can still bind to mitochondrial cyclophilin D) (*11*), and bongkrekic acid (BA), which binds to another site of the ANT than Atr (*13*) (Fig. 1A). These three inhibitors also prevented the effects of Bax on isolated mitochondria: $\Delta\Psi_m$ reduction (Fig. 1, B and C), release of cytochrome c (Fig. 1E), and release of a factor different from cytochrome c (*2*) that causes isolated nuclei to fragment their DNA in a cell-free system [apoptosis-inducing factor (AIF)] (Fig. 1D). In contrast, the caspase inhibitor z-VAD.fmk did not inhibit the $\Delta\Psi_m$ dissipation induced by Bax microinjection, although it did inhibit nuclear apoptosis. Bax-mediated effects on mitochondria and nuclei also did not require de novo protein synthesis (Fig. 1A). Thus, the effect of Bax on mitochondria involves a close functional interaction with the pharmacological targets of BA and CsA.

The PTPC contains several constitutive mitochondrial membrane proteins, including the inner transmembrane protein ANT and the soluble matrix protein cyclophilin D (*11, 12*). PTPC isolated from the rat brain normally contains Bax (*14*) and can be reconstituted into liposomes, where it regulates liposomal membrane permeability similarly to the PT pore in intact mitochondria (*14, 15*). Addition of the hydrophilic ANT ligand Atr (which binds to the intermembrane face of the ANT) (*13*) caused liposomes containing PTPC (*16*) to release small molecules such as [^{3}H]glucose (180 daltons, Fig. 2A) or $DiOC_6(3)$ (573 daltons, Fig. 2, C and D), but not [^{3}H]inulin (5200 daltons, Fig. 2B), indicating that PTPC liposomes contain ANT with the Atr-binding site at the surface, which is in the same orientation as in mitoplasts. When Bax was immunodepleted from PTPC before PTPC reconstitution into liposomes, the resulting complex failed to respond to Atr (Fig. 2, A and D). This cannot be due to depletion of the Atr target because Bax-immunodepleted PTPC still contained ANT molecules (Fig. 2C) and because recombinant Bax (but not recombinant Bcl-2) added to Bax-depleted PTPC could restore a CsA- or BA-inhibitable response to Atr (Fig. 2D). Moreover, this is a selective effect, because the Bax-depleted PTPC continued to respond to the reactive oxygen species donor *tert*-butylhydroperoxide (*t*-BHP) (Fig. 2, A and D), the thiol-cross-linking agent diamide (Fig. 2D), and caspases 1 or 3 (*17*). Bcl-2 prevented the *t*-BHP–induced membrane permeabilization, even when Bax was depleted from the PTPC (Fig. 2D), in accord with the fact that Bcl-2 can exert at least some of its death-antagonistic effects in the absence of Bax (*18, 19*).

When purified from the brain of Bax-deficient ($Bax^{-/-}$) mice and reconstituted into liposomes, PTPC did not respond to Atr, whereas PTPC from control mice ($Bax^{+/+}$) responded normally (Fig. 3A). Again, this defect is selective for Atr (but not *t*-BHP or diamide) and could be reverted by the addition of Bax (but not the inactive Bax mutants ΔIGDE or Δα5/6; Fig. 3A), thus confirming the data obtained with Bax-immunodepleted rat PTPC. In addition, Atr did not disrupt the $\Delta\Psi_m$ of liver mitochondria purified from $Bax^{-/-}$ mice in conditions in which it caused a $\Delta\Psi_m$ collapse in mitochondria from control ($Bax^{+/+}$) mice (Fig. 3B). Further genetic evidence for the Bax-ANT cooperation was obtained in yeast (*Saccharomyces cerevisiae*) cells in which all three genes coding for ANT isoenzymes were inactivated. Transfection of wild-type yeast with galactose-inducible Bax plasmid resulted in markedly reduced colony formation on galactose plates (<1% survival after 24 hours of Bax overexpression), con-



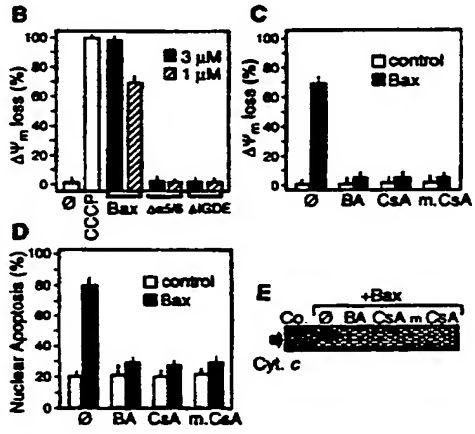


Fig. 1. Bax effects are neutralized by inhibitors of the PT pore. (**A**) Microinjection of Bax or Atr induces $\Delta\Psi_m$ dissipation and nuclear apoptosis in a BA-inhibitable fashion. Rat-1 fibroblast cells were microinjected with Atr (50 μM), Bax (1 μM), mutant Bax proteins (ΔIGDE or Δα5/6), or buffer only. BA (50 μM), CsA (1 μM), m.CsA (1 μM, SDZ 220-384; Novartis, Basel, Switzerland), the caspase inhibitor z-VAD.fmk (50 μM; Bachem, Basel Switzerland), or the protein synthesis inhibitor cycloheximide (CHX, 35 μM) were added to the culture medium 30 min before microinjection into the cytoplasm (pressure 150 hPa; 0.2 s). After microinjection, cells were cultured for 90 min and stained for 10 min with the potential-sensitive dye CMXRos [100 nM, red fluorescence; negative control obtained with 50 μM protonophore (CCCP) added before staining] and the DNA-intercalating Hoechst 33258 dye (1 μM, blue fluorescence). Microinjected viable cells (100 to 200 per session, two to four independent sessions of injection) were identified by inclusion of 2.5% (w/v) fluorescein isothiocyanate–labeled dextran (green fluorescence) in the injectate (not shown). Representative microphotographs of microinjected cells are shown. (**B**) Bax causes $\Delta\Psi_m$ reduction in isolated mitochondria. Bax or its mutants (ΔIGDE or Δα5/6) were added to purified (*32*) mouse liver mitochondria (1 mg of protein per milliliter) resuspended in 220 mM sucrose, 68 mM mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 500 μM EGTA, 5 mM succinate, 2 μM rotenone, 10 mM Hepes (pH 7.2). The $\Delta\Psi_m$ was monitored by measuring the quenching of rhodamine 123 fluorescence (5 μM) (*5*). The protonophore CCCP (10 μM) served as positive control (100%) of $\Delta\Psi_m$ dissipation (mean ± SD of triplicates; representative of six experiments). 198, negative control. (**C**) Inhibitory effect of BA (50 μM), CsA (1 μM), and m.CsA (1 μM) on the Bax-induced (1 μM, 60 min) $\Delta\Psi_m$ dissipation. The $\Delta\Psi_m$ dissipation was measured as in (B). (**D**) Inhibitory effect of BA, CsA, or m.CsA on the mitochondrial release of apoptosis-inducing factor. The supernatant of mitochondria treated with Bax or PT pore inhibitor, or both as in (C) was added to isolated HeLa nuclei, and DNA loss occurring after 90 min of incubation was assessed by cytofluorometric analysis of propidium iodide–stained nuclei (*33*). (**E**) Bax-induced cytochrome c release and its inhibition by BA, CsA, and m.CsA (Co., control). The supernatants of mitochondria incubated as in (D) were subjected to immunoblot analysis (*34*).

sistent with previous reports that Bax kills yeast cells through a mitochondria-targeting mechanism (*9, 20*). In contrast, ANT-deficient yeast were completely resistant to Bax, forming colonies comparable to cells transformed with control plasmids (Fig. 3C). These data emphasize the functional interaction between Bax and the Atr target ANT.

In mammalian cells, Bax and ANT coimmunoprecipitated (Figs. 2C and 4A), thus supporting our observation that Bax copurified with PTPC, which contains ANT protein (*14*) (Fig. 2C). We observed the interaction between ANT and Bax in different tissues including rat brain (Fig. 2C), mouse liver, and human HT-29 colon cancer cells (Fig. 4A). In this latter cell line, ANT and Bcl-2 also coimmunoprecipitated (*21*) (Fig. 4A). This interaction is likely to be direct, on the basis of two observations. First, in a yeast two-hybrid system (*22*) a fragment (amino acids 105 to 156) of the ubiquitously expressed human ANT2 isoenzyme interacted with Bax and with its close homolog Bak as well as with Bcl-2 (Fig. 4B). Second, recombinant Bax and purified ANT cooperated to reproduce some features of the PTPC in vitro. For the latter experiments, ANT was purified to >95% homogeneity and found to be uncontaminated by other proteins typically contained in PTPC (cyclophilin D, porin, and Bax) (*23*). When incorporated into liposomal membranes, only the combination of purified ANT and Bax, but neither molecule alone, efficiently permeabilized the liposomal membranes in the presence of Atr (Fig. 4C). Thus, in these conditions, ANT and Bax are necessary and sufficient to form Atr-sensitive pores in artificial membranes. It is of note that in this system Bax cannot be replaced by Bcl-2, Bax ΔIGDE, or Bax Δα5/6 (Fig. 4C), supporting the idea that homodimerization of Bax (deficient in Bax ΔIGDE) and pore formation (deficient in Bax Δα5/6) are required for the proapoptotic effect of Bax (*6–8, 10*).

When incorporated into artificial membranes, relatively large amounts of Bax can

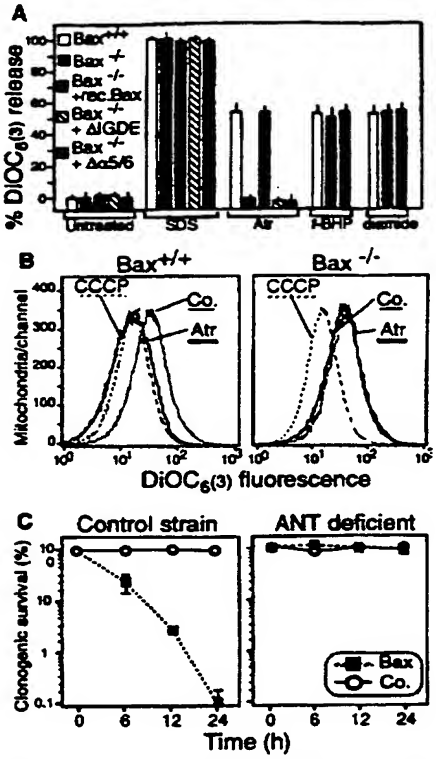


Fig. 3. Genetic evidence for the functional interaction between Bax and ANT. (**A**) Comparison of purified PTPC from wild-type ($Bax^{+/+}$) or Bax-deficient ($Bax^{-/-}$) mice. PTPC was extracted from the brains of $Bax^{+/+}$ or $Bax^{-/-}$ mice, reconstituted into liposomes, and tested for a response to Atr (100 μM), *t*-BHP (200 μM), or diamide (100 μM), followed by determination of $DiOC_6(3)$ retention as in Fig. 2D. Bax protein or two inactive mutant Bax proteins (ΔIGDE or Δα5/6) were added to PTPC from $Bax^{-/-}$ animals to restore the Atr response. (**B**) Failure of mitochondria from $Bax^{-/-}$ mice to respond to Atr. Isolated hepatocyte mitochondria from $Bax^{+/+}$ or $Bax^{-/-}$ animals were treated with Atr (5 mM) or the protonophore CCCP (50 μM). The $\Delta\Psi_m$ was measured with the potential-sensitive fluorochrome chloromethyl-X-rosamine (CMXRos) (100 nM, 15 min, room temperature) and analysis in a FACS Vantage cytofluorometer (Becton Dickinson, ~5 × 10^4 mitochondria per curve). Similar results were obtained when, instead of CMXRos, rhodamine 123 dequenching (CCCP = 100 %, control = 0%) was measured as in Fig. 2B (92 ± 4% and 7 ± 3% for Atr-treated $Bax^{+/+}$ and $Bax^{-/-}$ mitochondria, respectively, mean ± SEM of triplicates). Results are representative of four experiments on three independent mitochondrial preparations ($P < 0.001$, Student's *t* test). (**C**) Failure of Bax to kill ANT-deficient yeast cells. Wild-type yeast cells or cells lacking all three ANT genes were transfected with a galactose-inducible vector pJG-4-5-Bax (or pJG-4-5-vRas as a control, Co.). Bax or vRas expression was induced by switching culture conditions from a glucose-containing to a galactose-containing medium for the indicated period, and the clonogenic potential of yeast cells was assessed (20). Results are expressed as mean ± SEM of triplicates (SEM bars mostly obscured by symbols) and were reproduced twice.

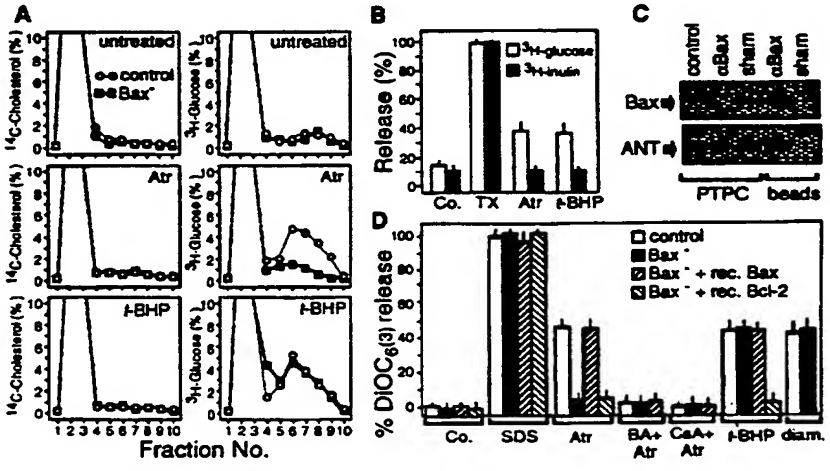


Fig. 2. Bax immunodepletion from PTPC abolishes the Atr-induced permeabilization of liposomal membranes. PTPC liposomes were immunodepleted from Bax, and their functional behavior was compared with control liposomes, which did contain Bax. (**A**) Bax depletion abolishes the Atr-induced release of [^{3}H]glucose. PTPC immunodepleted of Bax (black squares) or sham-immunodepleted (open circles) was incorporated into liposomes generated in the presence of [^{14}C]cholesterol (which incorporates into membranes) and [^{3}H]glucose (which marks the aqueous lumen), followed by addition of Atr (500 μM) or *t*-BHP (200 μM), molecular sieve chromatography, and determination of the radioactivity in liposomes (fractions 1 to 4) and the extraliposomal fluid (fractions 6 to 10). (**B**) Selectivity of the PTPC. Either [^{3}H]glucose or [^{3}H]inulin was encapsulated into control proteoliposomes. Then, the release of either of the radioactive substances [sum of factions 6 to 10 as in (A)] was determined as in (A) in response to the detergent Triton X-100 (TX, final concentration 2%), Atr (500 μM), or *t*-BHP (200 μM). (**C**) Immunodepletion of Bax from PTPC. PTPC was enriched by anion exchange chromatography, followed by immunodepletion of Bax or sham-immunodepletion with protein A and G agarose beads. The proteins remaining in PTPC or those attached to agarose beads were subjected to immunodetection of Bax or ANT. (**D**) Bax depletion selectively blocks the Atr-triggered release of $DiOC_6(3)$ from PTPC proteoliposomes. Bax-immunodepleted (Bax^-) or control PTPC liposomes were treated with Atr [100 μM; as in (D)], *t*-BHP (200 μM), diamide (diam.) (100 μM), CsA (1 μM), or BA (50 μM), equilibrated with $DiOC_6(3)$, and examined for $DiOC_6(3)$ retention in the cytofluorometer (rec., recombinant). Triplicates of 5 × 10^4 liposomes were analyzed and results were expressed as percent of reduction of $DiOC_6(3)$ fluorescence, considering the reduction obtained with 0.25% SDS in PTPC liposomes as 100% value, as described (*18*). Results are representative of three to five independent determinations.

form channels with several levels of conductance and little if any ion selectivity (*24*). Similarly, the purified ANT molecule can form nonspecific channels in artificial membranes in the presence of long-chain fatty acids or Ca^{2+} (*23, 25*). At the concentrations used here, the ANT-Bax complex on its own did not enhance the permeability of membranes unless Atr was supplied. This is compatible with evidence that Bax, though present in many cell types, does not trigger apoptosis on its own but rather increases the susceptibility of cells to the induction of apoptosis by exogenous stimuli (*26*).

Atr facilitated the ANT-Bax–dependent increase in membrane permeability (Figs. 2 to 4), whereas BA had the opposite effect (Figs. 1 and 2D). Atr binds to the external adenosine diphosphate (ADP)–binding site of ANT and locks it in the "c-state," which is compatible with PT pore opening. In contrast, BA binds to the internal adenosine triphosphate (ATP)–binding site of ANT and locks it in the "m-state," which prevents PT pore opening in response to a wide array of agents (*2, 12, 13*). Thus, the ANT conformation evidently determines pore formation by the Bax-ANT complex. The endogenous modulators of ANT conformation may include ATP/ADP ratios, Ca^{2+}, cellular redox status, and fatty acid metabolites (*23, 25, 27, 28*). By analogy to some receptor complexes, the PTPC could be organized into several functional units, which are responsible for distinct biological responses. In this scenario, interacting pairs of ANT-Bax molecules would form one particular pore-forming unit. Additional units regulated by other physiological effectors could arise from interactions of ANT (or ANT-like mitochondrial solute carriers) with outer membrane proteins, including porin (*14, 28*) or perhaps proteins of the mitochondrial protein import machinery (*29*). Several ANT-like proteins can form large conductance pores (*30*) and are targets of diamide (*11, 31*), a PT pore–opening agent not influenced by Bax (Figs. 2D and 3A) or Bcl-2 (*14, 15*). Hence, it is possible that different combinations of inner and outer membrane proteins simultaneously regulate mitochondrial membrane permeability and respond to distinct proapoptotic signal transduction or damage pathways.

Given that both ANT and Bax can form pores (*23–25*), it is conceivable that the ANT-Bax complex could form a gap junction–like conduit at the inner-outer membrane contact site or regulate the permeability of the outer and inner mitochondrial membranes in a coordinate fashion, or both. Alternatively, under some circumstances, Bax could translocate to and act primarily on the inner membrane in a colicin-like fashion. This latter possibility is underscored by immunoelectron microscopy data indicating that, upon treatment with Atr, Bax redistributes from the outer to the inner mitochondrial membrane. This redistribution has a diffuse rather than clustered pattern and is inhibited by CsA (Fig. 4D). Moreover, when added to mitoplasts, Bax and Bcl-2, but not the mutant proteins BaxΔα5/6or Bcl-2Δα5/6, interacted with the mitochondrial inner membrane in a CsA-inhibitable fashion (Fig. 4E), suggesting that the interaction of Bax with the inner membrane depends on the conformation of the PTPC. A subtle derangement in volume homeostasis of the mitochondrial matrix facilitated by Bax could provoke the local mechanical disruption of the outer membrane, as this has been suggested by the study of mitochondrial ultrastructure in apoptotic cells (*5*). This may explain why opening of the PT pore, which does not allow for the transit of molecules like inulin (5200 daltons; Fig. 2B) or cytochrome c (14 kD) (*14*), can trigger the release of intermembrane proteins through the outer membrane. Alternatively, or in addition, Bax may undergo ANT-dependent conformational changes or oligomerization events that permit it to form protein-translocating channels in the outer membrane (*8*).

In conclusion, PTPC may contain several channel-forming proteins that cooperate to mediate apoptosis responses. One such pair of cooperating proteins comprises the constitutive mitochondrial inner membrane protein ANT and the proapoptotic Bcl-2 analog Bax. This discovery provides a biochemical and mechanistic link between the PT pore and the mode of action of apoptosis regulatory proteins of the Bcl-2–Bax family.

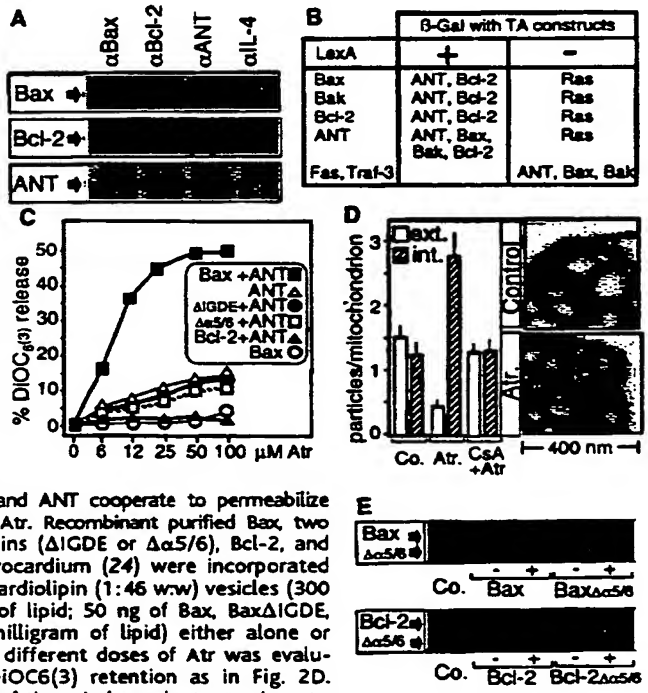


Fig. 4. Physical and functional interaction between Bax and ANT. (**A**) Coimmunoprecipitation of Bax and ANT. Triton-solubilized mitochondria from HT-29 cells were precipitated with antibodies (designated by α) specific for Bax, Bcl-2, ANT, or human IL-4, followed by protein immunoblot and immunodetection of Bax, Bcl-2, or ANT, as indicated. (**B**) Physical interaction between the ANT (human ANT2, amino acids 105 to 156) and Bax, as revealed by a yeast two-hybrid system. β-Galactosidase activity indicative of positive interactions and negative results, are listed. (**C**) Bax and ANT cooperate to permeabilize membranes in response to Atr. Recombinant purified Bax, two inactive mutant Bax proteins (ΔIGDE or Δα5/6), Bcl-2, and purified ANT from rat myocardium (*24*) were incorporated into phosphatidylcholine-cardiolipin (1:46 w:w) vesicles (300 ng of ANT per milligram of lipid; 50 ng of Bax, BaxΔIGDE, BaxΔα5/6, or Bcl-2 per milligram of lipid) either alone or together. The response to different doses of Atr was evaluated by measuring the DiOC6(3) retention as in Fig. 2D. Results are representative of three independent experiments. (**D**) Submitochondrial redistribution of Bax upon treatment with Atr. Purified mouse liver mitochondria were incubated during 30 min with 5 mM Atr or 1 μM CsA or both, fixed, and subjected to immunoelectron microscopy for the detection of Bax [anti-Bax plus Immunogold (5 nm) anti-rabbit immunoglobulin conjugate]. Representative micrographs counterstained with uranium acetate are shown. The number of grains located on the internal (hatched bars) or external (white bars) mitochondrial membrane were counted. Numbers indicate the number of grains per mitochondrion (mean ± SEM, n = 100; background value obtained with preimmune serum <0.1). (**E**) Interaction of Bax and Bcl-2 protein with the inner mitochondrial membrane. Mouse liver mitoplasts (*35*) (50 μg of protein in 50 μl; same buffer as in Fig. 1B) were incubated in the absence (Co.) or presence of 1 μM Bax, Bax Δα5/6, Bcl-2, or Bcl-2 Δα5/6 protein (30 min, 37°C), in the presence or absence of 1 μM CsA (denoted by + or −), washed (10 min, 10,000*g*, 4°C) three times, subjected to SDS-PAGE and immunodetection of Bax or Bcl-2 with antibodies recognizing both the native and the Δα5/6 proteins.

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10. Recombinant human Bcl-2(1–218) or mouse Bax(1–171) proteins lacking the hydrophobic transmembrane (TM) domain [deletion of amino acids 219 through 239 (Δ219–239) in the case of Bcl-2; Δ172–192 for Bax], Bcl-2Δα5/6[Bcl-2(Δ143–184)], Bax ΔIGDE (Δ66–69), and Bax Δα5/6 (Δ106–153) were created with a two-step polymerase chain reaction (PCR) method [H. Zha *et al.*, *Mol. Cell. Biol.* 16, 6494 (1996)]. These five proteins were then tagged with His_6 or glutathione *S*-transferase (in the case of Bax Δα5/6) and purified as described [(*8*); S. Schendel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94, 5113 (1997); Z. H. Xie, S. Schendel, S. Matsuyama, J. C. Reed, *Biochemistry* 37, 6410 (1998)].
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16. PTPC was prepared from Wistar rat brains or from $Bax^{-/-}$ [C. M. Knudson, K. S. Tung, W. G. Tourtellotte, G. A. Brown, S. J. Korsmeyer, *Science* 270, 96 (1995)] or $Bax^{+/+}$ C57Bl/6 mouse brains (*14*). For Bax immunodepletion the PTPC preparation was incubated with polyclonal antibody to Bax (anti-Bax; 50 μg/ml) (Δ21, Santa-Cruz) for 2 hours at room temperature. Sham immunodepletion was done with a preimmune rabbit antiserum. A 0.1 volume of protein A and protein G agarose beads was added for 30 min and removed by centrifugation (10 min, 2000*g*). PTPC in the supernatant (~1 μg of protein per milligram of lipid) was reconstituted into lipid vesicles (optionally supplemented with 0.5 μCi of [^{14}C]cholesterol per 100 mg of lipids) by overnight dialysis. Recombinant proteins were added during the dialysis step at a dose corresponding to 1% (Bax or Bax mutants) or 5% (Bcl-2) of total PTPC proteins. Liposomes were ultrasonicated in 5 mM malate and 10 mM KCl (optionally supplemented with 5 μCi of [^{3}H]glucose per milliliter or 5 μCi of [^{3}H]inulin per milliliter) and separated on Sephadex G50 columns (*14*). Proteoliposomes containing maximum hexokinase activity (*14*) were incubated (60 min, room temperature) with PT pore–opening agents in 125 mM sucrose plus 10 mM Hepes (pH 7.4). When the release of [^{3}H]glucose or [^{3}H]inulin was assessed, liposomes were separated on Sephadex G50 Nick columns (Pharmacia). Alternatively, liposomes were equilibrated for 30 min with 80 nM 3,3′-dihexylocarbocyanine [$DiOC_6$(3)] and then analyzed in a cytofluorometer (*14*).
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20. The *S. cerevisiae* W301-1B control strain (*MATα, ade2, trp1, leu2, ura3, his3*) and the triple ANT mutant JL-1-3 strain (*MATα, LEU::aac1, HIS::aac2, URA::aac3, ade2, trp1, leu2, ura3, his3*) [T. Drgon, L. Sabova, N. Nelson, J. Kolarov, *FEBS Lett.* 289, 159 (1991)] were transfected with a pJG–4-5 vector containing the Trp1 selection marker and murine Bax under the control of the Gal promoter, and the lethal effect of Bax overexpression (controlled by immunoblot) was assessed [S. Matsuyama, Q. Xu, J. Velours, J. C. Reed, *Mol. Cell* 1, 327 (1998)]. The pJG–4-5 vector containing v-Ras instead of Bax served as a negative control.
21. Cells or organs were homogenized in H buffer (150 μM $MgCl_2$, 10 mM KCl, 10 mM tris-HCl, pH 7.6), followed by elimination of nuclei (3 min at 900*g*, at 4°C, recovery of supernatant), elimination of soluble proteins (10 min at 6800*g*), resuspension of the mitochondria-containing pellet in H buffer supplemented with 0.5% Triton (v.v, final concentration; 0.5 mg of protein per milliliter), addition of polyclonal rabbit antiserum (1 μg of antibody per 10 μg of protein in sample) specific for Bcl-2, Bax (Santa Cruz Biotechnology), human interleukin-4 (IL-4) (Pharmingen), or ANT [J. Gironcalle and H. H. O. Schmid, *Biochemistry* 35, 15440 (1996)], incubation for 90 min at 37°C, addition of 10% protein A and G agarose beads (Santa Cruz Biotechnology) for 30 min at 37°C, recovery (10 min, 2000*g*) of the washed (two times in phosphate-buffered saline, pH 7.4) beads in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and immunodetection with monoclonal antibody (mAb) to Bax (P19, Santa Cruz), mAb to Bcl-2 (clone 124, Dako), or antiserum to ANT.
22. A human Bak cDNA without TM [J. M. Jürgensmeier *et al.*, *Mol. Biol. Cell* 8, 325 (1997)], human Bcl-2 lacking the TM, murine Bax cDNA lacking the TM, Ras, Fas, Traf-3, or a human ANT2 cDNA fragment (amino acids 105 to 156) were subcloned in the Eco R1–Xho I sites of pGilda or pJG4-5. EGY48 cells were used for *lacZ* reporter gene assays in conjunction with pGilda (LexA DNA–binding domain) and pJG4-5 (B42 transactivation domain) and pSH 18-34 reporter plasmids. Filter assays were performed for galactosidase measurement, with cells plated on either galactose- or glucose-containing minimal medium (*19*) [M. Hanada, C. Aime-Sempé, T. Sato, J. C. Reed, *J. Biol. Chem.* 270, 11962 (1995); H. Zha, C. Aimé-Sempé, T. Sato, J. C. Reed, *ibid.* 271, 7440 (1996)].
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Dorsal-Ventral Signaling in the *Drosophila* Eye

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The development of the *Drosophila* eye has served as a model system for investigations of tissue patterning and cell-cell communication; however, early eye development has not been well understood. The results presented here indicate that specialized cells are established along the dorsal-ventral midline of the developing eye by Notch-mediated signaling between dorsal and ventral cells, and that Notch activation at the midline plays an essential role both in promoting the growth of the eye primordia and in regulating eye patterning. These observations imply that the developmental homology between *Drosophila* wings and vertebrate limbs extends to *Drosophila* eyes.

Patterning and growth of the *Drosophila* wing depend on signaling between cells in different compartments (*1*, *2*). This signaling establishes specialized cells along compartment boundaries, which in turn secrete molecules that regulate wing development. The *fringe* (*fng*) gene regulates signaling between dorsal and ventral cells (*3*). Fringe protein (FNG) is produced by dorsal cells and inhibits cells' responsiveness to the Notch ligand Serrate (SER), while potentiating cells' responsiveness to the Notch ligand Delta (DL) (*4*, *5*). This results in activation of Notch along the *fng* expression border, which normally corresponds to the dorsal-ventral (D-V) compartment boundary. Local activation of Notch in the wing is both necessary and sufficient for long-range effects on the fate and proliferation of wing cells (*1*, *2*).

Strict compartment boundaries have not

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